Characterization of the charge variants of L2 β -lactamase in *Stenotrophomonas maltophilia*

Rouh-Mei Hu,¹ Kai-Hung Chiang,² Yi-Chih Chang²
and Tsuey-Ching Yang²

Correspondence
Tsuey-Ching Yang
tcyang@mail.cmu.edu.tw

¹Department of Biotechnology and Bioinformatics, Asia University, Taichung 413, Taiwan, ROC

²Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung 404, Taiwan, ROC

Stenotrophomonas maltophilia KH has two acid β -lactamases with isoelectric points (pIs) of 4.6 and 5.4, and several basic β -lactamases (pIs >7.0) that produce a ladder-shaped pattern by IEF. An isogenic L2 mutant, KHL2_{xyIE}, was constructed by gene replacement. From IEF and native PAGE zymograms of strains KH and KHL2_{xyIE}, it was demonstrated that the basic β -lactamases and the acid β -lactamase with pI 5.4 are encoded by the same L2 gene and that the active types of these L2 charge variants were dependent on the buffer pH. The β -lactamase activities of these L2 charge variants in phosphate buffer at pH 7.0 and 8.0 were 1075 ± 29 and 1114 ± 81 U mg⁻¹, respectively. These results indicate that L2 charge variants give *S. maltophilia* a better chance of adapting and surviving in response to changes in the environment.

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INTRODUCTION

Stenotrophomonas maltophilia, an aerobic, non-fermentative, Gram-negative bacillus, is ubiquitous in environments such as water, soil and sediments (Bollet *et al.*, 1995; Papapetropoulou *et al.*, 1994). Recently, it has emerged as a significant nosocomial pathogen capable of causing respiratory, urinary and bloodstream infections in hospitalized patients (Denton & Kerr, 1998).

S. maltophilia intrinsically produces at least two types of β -lactamase, known as L1 and L2 (Saino *et al.*, 1982, 1984), giving rise to resistance against virtually the entire spectrum of β -lactams (Alonso & Martinez, 1997). The isoelectric point (pI) of a β -lactamase is an important property used for characterization and classification (Huovinen, 1988). In general, L1 and L2 can readily be discriminated by their pI values, as determined by IEF, where L1 has a pI <7.0 and L2 has a pI >7.0 (Denton *et al.*, 1999). However, minor β -lactamase-active bands have been reported in IEF gels that have not yet been classified precisely (Bicknell *et al.*, 1985; Paton *et al.*, 1994). In this study, a clinical *S. maltophilia* isolate, KH, was found to display a peculiar IEF zymogram pattern, with two acid β -lactamases and several basic β -lactamases that formed a ladder-shaped pattern on the gel. Genetic and phenotypic characterization of the latter was also undertaken.

METHODS

Bacterial strains and media. Twenty isolates of *S. maltophilia* were investigated in this study. They were identified using an ID32 GN system (bioMérieux) and confirmed by PCR amplification of a 531 bp fragment in the 16S rRNA gene region (Whitby *et al.*, 2000). Unless otherwise specified, *Escherichia coli* and *S. maltophilia* were routinely cultured in Luria–Bertani (LB) medium and on L agar at 37 °C. The growth of *S. maltophilia* was monitored by measuring the OD₄₅₀ of the cultures.

Preparation of β -lactamase extracts. Twenty millilitres of fresh LB broth was inoculated with bacteria at a turbidity of 0.15 OD₄₅₀ after overnight growth at 37 °C. The culture was further incubated for 0.5 h. Cefoxitin (50 µg ml⁻¹) was then added and the incubation process was continued for 2.5 h. Bacterial cells from 20 ml of the culture were harvested by centrifugation (5 min, 4 °C, 3500 g) and washed with 50 mM sodium phosphate buffer at pH 7.0 or 8.0. Following resuspension of the pellet in 2 ml of the same buffer, the cells were disrupted by sonication. Cell debris was pelleted by centrifugation (30 min, 4 °C, 12 000 g) and the supernatant was transferred to a clean tube, ready for IEF, native PAGE and β -lactamase activity assays.

IEF. IEF analysis was performed using an ampholine polyacrylamide gel with a pH range of 3.5–9.5 (Ampholine PAGplate; Amersham Pharmacia Biotech) for 90 min at 1500 V, 50 mA and 25 W in a Multiphor II Electrophoresis System (Amersham Biosciences; Matthew & Harris, 1976; Mathew *et al.*, 1975). The separated β -lactamases were detected by overlaying the gel with 100 µM nitrocefin solution (Oxoid). The pI of each β -lactamase was determined using a pH 3–10 broad-range pI calibration kit (Amersham Biosciences).

Construction of the gene replacement plasmid pKHL2_{xyIE}. According to *S. maltophilia* K279a genome sequences (www.sanger.ac.uk/Projects/S_maltophilia; Crossman *et al.*, 2008), an *ampR* homologue gene, denoted here as *ampR*, was located separately

Abbreviation: pI, isoelectric point.

The GenBank/EMBL/DDBJ accession number for the sequence of the *ampR* gene, intergenic region and L2 β -lactamase gene is EU032534.

upstream of the L2 gene. We designed the primers AmpRL2-F (5'-CGACCAACCACCTTGACC-3') and AmpRL2-R (5'-ATGCCGATGATGCCGAAC-3') to amplify these two genes together (*ampR*-L2) from *S. maltophilia* KH. PCRs were conducted in a reaction volume of 50 μ l containing PCR buffer [500 mM KCl, 1% Triton X-100, 100 mM Tris/HCl (pH 9.0), 2.5 mM MgCl₂], 200 μ M each dNTPs, 10% DMSO, 2 U SuperTaq XL (ProTech), 1.5 μ M primer for each reaction and 2 μ l DNA template. The PCR conditions were 94 °C for 5 min, followed by 30 cycles of 60 s at 94 °C, 60 s at 58 °C and 90 s at 72 °C, with a final 10 min extension step at 72 °C. A 2.8 kb PCR product was ligated into T-vector (Yeastern Biotech), giving rise to pTKHRL2. A catechol 2,3-dioxygenase gene cassette, *xylE*, was retrieved from pX1918GT (Schweizer & Hoang, 1995) by PCR with the primer pair XylE-F (5'-GAATTCGCGGCCGCGATCAAGGAC-TACGATT-3') and XylE-R (5'-GCGGCCGCAAGTCGTACCGGAC-CATCAG-3') and was inserted into the *Stu*I site of the L2 gene. This disrupted the reading frame of the L2 gene in pTKHRL2. The orientation of *xylE* was determined by sequencing to see whether it was the same as that of the L2 gene. Subsequently, the DNA fragment of L2::*xylE* from pTKHRL2::*xylE*, as a *Hind*III/*Bgl*II-cut fragment, was subcloned into pEX18Tc, creating pKHRL2_{*xylE*}. This plasmid was transformed into *E. coli* S17-1 to produce the donor strain *E. coli* S17-1(pKHRL2_{*xylE*}) ready for conjugation.

Conjugation. An overnight culture of each bacterium to be mated was prepared using LB broth. The cultures of donor strain [*E. coli* S17-1(pKHRL2_{*xylE*})] and recipient strain (*S. maltophilia* KH) were mixed at a ratio of 10:1. These mixed cultures were spread onto nitrocellulose membrane in the centre of a nutrient agar plate (no antibiotics added) and incubated at 37 °C overnight. The merodiploids from homologous recombination were selected on LB agar containing tetracycline (40 μ g ml⁻¹) and norfloxacin (2.5 μ g ml⁻¹). Transconjugants were then streaked onto LB agar containing 10% sucrose, and the resultant sucrose-resistant colonies formed after overnight incubation at 37 °C were screened for mutants.

Determination of cefepime MICs. MICs for cefepime were quantified using Etest strips (AB Biodisk) according to the manufacturer's instructions. The MIC was defined as the lowest concentration of an antibiotic that prevented visible growth of bacteria. *E. coli* ATCC 25922 was included as a control.

Native PAGE and in-gel activity staining. Native PAGE was performed according to standard SDS-PAGE protocols (Laemmli, 1970) without the addition of SDS. The gel was immersed in a 100 μ M nitrocefin solution to visualize the β -lactamase-active band.

β -Lactamase activity determination. β -Lactamase activity was determined kinetically by a UV spectrophotometric method as the change in A_{486} with 100 μ M nitrocefin as the substrate. The molar absorption coefficient for nitrocefin at 486 nm is 20 500 M⁻¹ cm⁻¹, as described by the manufacturer (Oxoid). One unit of β -lactamase activity was defined as the amount of enzyme that hydrolysed 1 nmol nitrocefin min⁻¹. Protein concentrations of clear lysates were determined using a Bio-Rad protein assay reagent, with BSA as the standard. The specific activity of the enzyme was defined in terms of units (mg protein)⁻¹ in cell extracts.

RESULTS AND DISCUSSION

IEF assay of *S. maltophilia* isolates

Two major discrete IEF patterns were observed among the 20 isolates: pattern I with two major bands and pattern II composed of multiple bands forming a ladder-shaped

pattern (see Fig. 1, lane 1, as a representative example of pattern II). Among the 20 isolates tested, 17 isolates exhibited pattern I and three had pattern II.

Pattern I revealed for these 17 isolates was typical of *S. maltophilia* β -lactamases, with one band of pI <7.0 (referred as L1) and the other band of pI >7.0 (assumed to be L2). In contrast, pattern II was rather unexpected and consisted of two acidic β -lactamases (pI <7.0) and a series of β -lactamases with pIs >7.0. We suggested two hypotheses for the formation of this ladder-like band pattern: (i) the different proteins were encoded by the same gene (presumed to be L2) and had undergone amino acid modifications, which, in turn, produced a series of charge variants; or (ii) this pattern of β -lactamases resulted from separate β -lactamase genes, implying that, in addition to the L1 and L2 genes, *S. maltophilia* contains other β -lactamase genes.

To test these hypotheses, an isolate with IEF pattern II, denoted *S. maltophilia* KH, was chosen for further study.

Sequence analysis of the L2 gene and *ampR* of *S. maltophilia* KH

Two divergently transcribed genes, L2 and *ampR*, were determined in the 2.8 kb PCR amplicons and were found to be separated by a 174 bp intergenic region. The G+C content of *ampR*, the intergenic region and the L2 gene was 70, 52 and 70 mol%, respectively. The G+C content of the intergenic region was notably low compared with the overall G+C content of *S. maltophilia* (66 mol%). The L2 gene encoded a 303 aa polypeptide whose N-terminal sequence exhibited features typical of prokaryotic signal peptides (<http://www.cbs.dtu.dk/services/SignalP/>).

Fig. 1. IEF of β -lactamase extracts from isolate KH and mutant KHL2_{*xylE*} in different buffer systems. pI values according to the pH 3–10 broad-range pI calibration kit (Amersham Biosciences) are indicated on the left. Lanes: 1 and 2, KH in phosphate buffer at pH 7.0 and 8.0, respectively; 3 and 4, KHL2_{*xylE*} in phosphate buffer at pH 7.0 and 8.0, respectively.

Characteristic elements of Amber class A β -lactamases were identified in the L2 protein, including the consensus residues STXK (aa 83–86), SDN (aa 143–145) and KTG (aa 247–249), and a glutamic acid residue (aa 179) located 34 residues downstream from the SDN loop (Walsh *et al.*, 1997). A sequence homology search using BLAST (<http://www.ncbi.nlm.nih.gov/>) revealed that L2 exhibited a sequence identity of 69–95 % with other L2 proteins from different *S. maltophilia* strains, and 55–58 and 52 % identity with β -lactamases of the genera *Xanthomonas* and *Bordetella*, respectively. The 288 aa AmpR protein shared 98 % identity with AmpR of *S. maltophilia* R551-3, 68–72 % identity with the known AmpR proteins from the genus *Xanthomonas* and 32–72 % identity with those of the genus *Pseudomonas*. AmpR had the characteristics of a typical LysR transcriptional regulator protein (Schell, 1993), with a helix–turn–helix DNA-binding motif in the N-terminal region (aa 23–43).

Genotypic and phenotypic confirmation of the isogenic L2 mutant KHL2_{xyIE}

The isogenic L2 mutant of isolate KH, KHL2_{xyIE}, was obtained by conjugation between *E. coli* S17-1(pKHRL2_{xyIE}) and *S. maltophilia* KH by two-step antibiotic/10 % sucrose selection. The mutant KHL2_{xyIE} was genetically verified by PCR/sequencing analysis and phenotypically confirmed by a susceptibility test against cefepime, which is known to be a susceptible substrate for L2 (Hu *et al.*, 2008). MIC values against cefepime for strain KH and mutant KHL2_{xyIE} were 32 and 2 $\mu\text{g ml}^{-1}$, respectively. As expected, a mutation in the L2 gene caused an obvious loss of resistance against cefepime.

The β -lactamases forming the ladder-shaped pattern are encoded by the same L2 gene

A pH 7.0 β -lactamase extract of mutant KHL2_{xyIE} was prepared as described above. IEF was carried out using the pH 7.0 extracts of KH and KHL2_{xyIE} (Fig. 1, lanes 1 and 3). The basic ladder-like pattern of β -lactamase bands and the band of pI 5.4 were absent in the extracts from mutant KHL2_{xyIE}, indicating that mutation of the L2 gene resulted in the loss of these additional β -lactamases. Therefore, hypothesis (i) proposed above appeared to be plausible in *S. maltophilia*.

Multiple β -lactamases on IEF gels have been described in the literature. When purifying the L1 protein by chromatofocusing, Bicknell *et al.* (1985) found three charge variants of L1 β -lactamases of *S. maltophilia* IID 1275. However, a genetic approach has not been attempted to elucidate the relationship of the charge variants. Four different β -lactamases were also reported from seven *S. maltophilia* isolates: an L1 of pI 6.8 and three β -lactamases with pI values of 6.2, 5.55 and 5.3 (Paton *et al.*, 1994). Other than their acidic pI values, these three β -lactamases share several similar features with the L2 enzyme. Nevertheless, in-depth exploration using a molecular

approach is still lacking in the literature. This study shows for the first time, to our knowledge, that the different β -lactamase charge variants are products of the same L2 gene.

The active types of the L2 charge variants are pH-dependent

In general, pH value is crucial for the occurrence of charge variants of a protein. To evaluate the effects of pH shift on the active types of the L2 charge variants, the β -lactamase extracts of isolate KH and mutant KHL2_{xyIE} were prepared in 50 mM sodium phosphate buffer at pH 7.0 or 8.0. As can be seen in Fig. 1, the active charge variants of L2 β -lactamase differed markedly when the buffer pH was shifted from 7.0 to 8.0. At pH 7.0, the pI values of the active L2 charge variants ranged from 5.4 to 7.9. More basic active L2 charge variants (pI 5.4–8.5) were revealed when the buffer pH was shifted to basic conditions. This trend was further confirmed by 10 % native PAGE followed by in-gel activity staining with nitrocefin (Fig. 2). Because the resolution of native PAGE for pI discrimination is inferior to that of IEF, all L2 charge variants in the native polyacrylamide gel were focused in a single band. Nevertheless, it was still obvious that the active L2 charge variants in the pH 8.0 buffer (Fig. 2, lane 2) exhibited lower mobility than those in the pH 7.0 buffer (Fig. 2, lane 1). The pI values of the active L2 charge variants also varied with the pH of the buffer system. Higher pI L2 charge variants appeared in more basic buffers. This phenomenon implies that some differential modification, folding and/or multimerization of the L2 enzyme occurred, either during gene expression or in response to the pH of the buffer.

Fig. 2. Native PAGE and in-gel activity staining by nitrocefin of β -lactamase extracts from isolate KH and mutant KHL2_{xyIE} in different buffer systems. L1 and L2 β -lactamases are indicated by arrows. Lanes: 1 and 2, KH in phosphate buffer at pH 7.0 and 8.0, respectively; 3 and 4, KHL2_{xyIE} in phosphate buffer at pH 7.0 and 8.0, respectively.

β -Lactamase activity assay of the different L2 charge variants

To elucidate whether the different L2 charge variants in 50 mM phosphate buffer at different pH values displayed different β -lactamase activity, the induced β -lactamase activities of strains KH and KHL2_{xylE} were determined. The assumed L2 β -lactamase activity was obtained by subtracting the activity of KHL2_{xylE} from that of KH. The β -lactamase activities of L2 in pH 7.0 and 8.0 buffers were approximately equal (1075 ± 29 vs 1114 ± 81). Thus the L2 charge variants exhibited a broad optimal pH range, i.e. 7.0–8.0. In previous reports, the purified L2 enzymes from *S. maltophilia* IID1275 and GN12873 displayed a sharp pH optimum at pH 7 and pH 7.5, respectively (Cartwright & Waley, 1984; Saino *et al.*, 1984). The pI values of the L2 enzymes from strains IID1275 and GN12873 are 9.3 and 8.4 (Cartwright & Waley, 1984; Saino *et al.*, 1984), suggesting that the L2 forms of strains IID1275 and GN12873 are likely to belong to pattern I of the IEF assay. In addition to pattern I, this study identified an additional type of L2 β -lactamase, denoted pattern II (Fig. 1, lanes 1 and 2), which exhibited different active types of charge variants under different pH conditions. These L2 charge variants of pattern II displayed a wider optimal pH range than that of pattern I.

S. maltophilia is widely distributed in different environments and geographical regions. The L2 enzyme shows a wide substrate profile for most β -lactam antibiotics, effectively protecting *S. maltophilia* from the effect of β -lactams. The existence of multiple L2 charge variants under different pH conditions gives the L2 enzyme high activity under different pH conditions, thus allowing *S. maltophilia* to adapt to fluctuating environments.

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REFERENCES

- Alonso, A. & Martinez, J. I. (1997). Multiple antimicrobial resistance in *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* **41**, 1140–1142.
- Bicknell, R., Emanuel, E. L., Gagnon, J. & Waley, S. G. (1985). The production and molecular properties of the zinc β -lactamase of *Pseudomonas maltophilia* IID 1275. *Biochem J* **229**, 791–797.
- Bollet, C., Davin-Regli, A. & De Micco, P. (1995). A simple method for selective isolation of *Stenotrophomonas maltophilia* from environmental samples. *Appl Environ Microbiol* **61**, 1653–1654.
- Cartwright, S. J. & Waley, S. G. (1984). Purification of β -lactamases by affinity chromatography on phenylboronic acid-agarose. *Biochem J* **221**, 505–512.
- Crossman, L. C., Gould, V. C., Dow, J. M., Vernikos, G. S., Okazaki, A., Sebaihia, M., Saunders, D., Arrowsmith, C., Carver, T. & other authors (2008). The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. *Genome Biol* **9**, R74.
- Denton, M. & Kerr, K. G. (1998). Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. *Clin Microbiol Rev* **11**, 57–80.
- Denton, M., Keer, V. & Hawkey, P. M. (1999). Correlation between genotype and β -lactamases of clinical and environmental strains of *Stenotrophomonas maltophilia*. *J Antimicrob Chemother* **43**, 555–558.
- Hu, R. M., Huang, K. J., Wu, L. T., Hsiao, Y. J. & Yang, T. C. (2008). Induction of L1 and L2 β -lactamases of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* **52**, 1198–1200.
- Huovinen, S. (1988). Rapid isoelectric focusing of plasmid-mediated β -lactamases with Pharmacia PhastSystem. *Antimicrob Agents Chemother* **32**, 1730–1732.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Mathew, A., Harris, A. M., Marshall, M. J. & Ross, G. W. (1975). The use of analytical isoelectric focusing for detection and identification of β -lactamases. *J Gen Microbiol* **88**, 169–178.
- Matthew, M. & Harris, A. M. (1976). Identification of beta-lactamases by analytical isoelectric focusing: correction with bacterial taxonomy. *J Gen Microbiol* **94**, 55–67.
- Papapetropoulou, M., Iliopoulou, J., Rodopoulou, G., Detorakis, J. & Paniara, O. (1994). Occurrence and antibiotic resistance of *Pseudomonas* species isolated from drinking water in southern Greece. *J Chemother* **6**, 111–116.
- Paton, R., Miles, R. S. & Amyes, S. G. N. (1994). Biochemical properties of inducible β -lactamases produced from *Xanthomonas maltophilia*. *Antimicrob Agents Chemother* **38**, 2143–2149.
- Saino, Y., Kobayashi, F., Inoue, M. & Mitsuhashi, S. (1982). Purification and properties of inducible penicillin β -lactamase isolated from *Pseudomonas maltophilia*. *Antimicrob Agents Chemother* **22**, 564–570.
- Saino, Y., Inoue, M. & Mitsuhashi, S. (1984). Purification and properties of an inducible cephalosporinase from *Pseudomonas maltophilia* GN12873. *Antimicrob Agents Chemother* **25**, 362–365.
- Schell, M. A. (1993). Molecular biology of the LysR family of transcriptional regulators. *Annu Rev Microbiol* **47**, 597–626.
- Schweizer, H. P. & Hoang, T. T. (1995). An improved system for gene replacement and xylE fusion analysis in *Pseudomonas aeruginosa*. *Gene* **158**, 15–22.
- Walsh, T. R., MacGowen, A. P. & Bennett, P. M. (1997). Sequence analysis and enzyme kinetics of the L2 β -lactamase from *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* **41**, 1460–1464.
- Whitby, P. W., Carter, K. B., Burns, J. L., Royall, J. A., Lipuma, J. J. & Stull, T. L. (2000). Identification and detection of *Stenotrophomonas maltophilia* by rRNA-directed PCR. *J Clin Microbiol* **38**, 4305–4309.